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Effect of Calcium Ionophore A23187 upon the Rate of Leukotriene C_4 Production and the Cellular Morphology in Highly Purified Mouse Peritoneal Macrophages Cultured *in vitro*

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ABSTRACT—When the highly purified mouse peritoneal macrophages cultured *in vitro*, were exposed to calcium ionophore, A23187, at a concentration of 0.5 μg/ml for 1.5–2.0 hr under alkaline conditions (pH 7.6–8.0) of the medium, the rate of leukotriene C_4 (LTC_4) production by these cells was enhanced approximately by 100 times. The amount of LTC_4 produced was measured by assaying the total amount of cysteinyl leukotrienes released into the medium: the newly developed monoclonal antibodies (supplied by Dr. F. Kohen, Dept. Hormone Res. Weizmann Inst., Rehovot, Israel), specific to cyteinyl leukotrienes were used for the purpose. Concomitantly, the ionophore transformed the macrophages of rounded cellular shape to assume the strongly elongated cellular morphology. Causal relationship between the two phenomena, however, remains to be investigated.

INTRODUCTION

In 1980, Samuelsson *et al.* [1, 2] introduced the term, leukotriene, to describe a family of compounds which are metabolites of arachidonic acid, produced via 5-lipoxygenase pathway and contain conjugated triene. Elucidation of the molecular structures of various leukotrienes opened an entirely new perspectives to the understanding of the mechanisms which underlie the functions of leukocytes, the regulation of the activities of smooth muscles, the pathogenesis of inflammation, allergy, asthma etc. (for reviews see [3–9]).

Leukotriene C_4 (LTC_4 in the following) is the first peptidolipid of the family to be synthesized in the pathway and an important component of SRS (slow reacting substance) and/or SRS-A (slow reacting substance of anaphylaxia) [3]. Both the peritoneal [10, 11] and the alveolar [12] macrophages cultured *in vitro* have been shown to produce LTC_4 in response to the stimulation by calcium ionophore A23187 [10–12]. Dependence of LTC_4 production upon the intracellular high concentration of Ca_2^+, i.e., $[Ca^{2+}]_i$, has been demonstrated in cultured leukemic basophils [13]; hydrocortisone reduces antigen-induced elevation of $[Ca^{2+}]_i$ and, as its consequence, the rate of LTC_4 production in these cells.

On the other hand, it has been known that cytoplasm of macrophages contains calcium-sensitive protein component, gelsolin, which is probably responsible for the regulation of the cytoskeletal organization of the cells [14–16], and that macrophages change their shape according to their functional states or to the modulation of environmental conditions (for reviews see [17, 18]). It might be possible, therefore, that the stimulation of LTC_4 production by calcium ionophore in the cultured macrophages, is correlated with the morphological changes of the cells.

To answer the question, we assayed the rate of LTC_4 production in the highly purified peritoneal macrophages of the mouse using newly developed monoclonal antibodies specific against cysteinyl leukotrienes, and, at the same time, observed the

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changes in the cellular morphology of the same cells. The results will be described in this paper. Summarized account of the findings were presented in abstract form [19].

MATERIALS AND METHODS

Animals

Adult mice of BALB/c (H-2^d) and C3H/HeJ (H-2^k) strains were purchased from a local dealer (Nippon Clea & Co., Ltd., Tokyo, Japan). They had been kept under regulated temperature (25°C) and illumination cycles (12 hr dark and 12 hr light per day), until the day of the experiments. Both male and female mice were used; they were 8–10 weeks old and the female mice were in diestrus of the estrous cycle at the time of the experiments.

Compounds and reagents

Tritium-labelled leukotriene C_4 (specific activity, 39 Ci/mMol) purchased from Amersham Japan & Co., Ltd. (Tokyo, Japan), was diluted with 50% ethanol to give a final concentration of 1.0 μCi/ml. Unlabelled LTC_4 (Wako Pure Chemical Industries & Co., Ltd., Osaka, Japan) was dissolved in double distilled water. Stock solution of calcium ionophore A23187 (Sigma Chemical & Co., Ltd., St. Louis, Mo, USA) was prepared by dissolving the compound in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries & Co., Ltd., Osaka, Japan) at a concentration of 500 μg/ml. Sterilized Ficoll-Hypaque solution ($d=1.090\pm0.001$) was purchased from Otsuka Assay Laboratories (Tokushima-shi, Japan).

Antibodies and antibody solution

Since LTC_4 may be rapidly converted into LTD_4 and LTE_4 by macrophages *in vitro,* as recently shown in the rat [20, 21], the amount of the three cysteinyl leukotrienes in the medium will have to be assayed simultaneously in order to assess correctly the rate of LTC_4 production by these cells. The monoclonal antibodies against the cysteinyl leukotrienes were generously supplied from Dr. F. Kohen, Department of Hormone Research, Weizmann Institute of Science, Rehovot, Israel: the antibodies react with LTC_4 (100%), LTD_4 (105%), and LTE_4 (77%) at a 50% saturation level of binding (F. Kohen, 1988, personal communication).

The stock solution of the antibodies was prepared by dissolving freeze-dried IgG in phosphate buffered saline containing heat-inactivated horse serum at a concentration of 3% (abbreviated in the following as PBS-HS/3), and stored at −20°C. Immediately before use, an aliquot of the stock solution was diluted with PBS-HS/3 to an appropriate level of the titre.

Macrophages

Macrophages were collected from peritoneal exudate of the mice following injection of Earl's minimum essential medium (MEM) containing heparin at a concentration of 30 units/ml [22]. The highly purified macrophage cell cultures were prepared, according to the procedures described elsewhere [23, 24], as follows. The lavage was layered onto a Ficoll-Hypaque gradient [25] and centrifuged at approximately 1200×g. A band formed between the lavage and Ficoll-Hypaque contains white cells including macrophages. The cells were removed, suspended in MEM fortified with foetal calf serum at a concentration of 20% (abbreviated in the following as MEM-FCS/20), and plated onto Falcon dishes. They were incubated for 1.0 hr at 37°C under an atmosphere of 5% CO_2 and 95% air. At the end of the incubation, the non-adherent cells were removed by decanting the medium. Then fresh MEM-FCS/20 was added to the cells. The medium, MEM-FCS/20, was used at the initial phase of the culture, because the presence of FCS at relatively high concentrations (20–40%) in the medium limits the adherence of B-lymphocytes to the culture dishes [26]. The remaining cells after the wash, were incubated for another 1 hr. Then the medium was replaced with fresh MEM-FCS/10. The incubation was continued for another 16 hr. Decantation of the medium with the detached cells, and replacement with the fresh FCS-MEM/10 were done as before. The cells removed from the dish at each step, mainly consisted of lymphocytes and dead macrophages; the number of the removed cells decreased sharply as the procedures were repeated. At the end of 16 hours' incubation, approximately

20% of the cells present in the original suspension, remained firmly adhered to the bottom surface of the dish: those cells represented very pure population of peritoneal macrophages. They were incubated in fresh MEM-FCS/10 for additional 24–48 hr to "stabilize" them under an atmosphere of 2-3% CO_2 and 97–98% air. Immediately before the start of the experiments where the rates of LTC_4 production were assayed, the medium was replaced with the freshly prepared MEM containing 4 mM cysteine without addition of FCS; the macrophages were incubated in the assay medium, either with or without the ionophore, for 1.5–2.0 hr under an atmosphere of 2–3% CO_2 and 97–98% air.

Radioimmunoassay procedure for LTC_4

One tenth ml of the sample solution to be assayed was mixed with an equal amount of the antibody solution and incubated at 0°C for 30 min. At the end of the incubation, 0.1 ml of ^3H-LTC_4 solution containing 7 nCi of the isotope in 50% ethanol-PBS, was added to the mixture, and had been stood at 4°C for overnight [27]. Free LTC_4 was removed by adding 0.2 ml of dextran-charcoal [27, 28], and by centrifugation at 15,000 rpm for 3 min. The radioactivity was measured by means of liquid scintillation counting.

Counting of macrophages according to their cell shape

The cells were fixed with 3.5% formaldehyde in 1/15 M phosphate buffer (pH 7.4), and stained with Giemsa's. The stained dishes were mounted on a projection microscope, and the magnified images were projected onto a digitizer tablet (model DT 1000, Watanabe Sokki, Tokyo, Japan). Then, the number of the cells of the elongated, or the rounded morphology was differentially counted. The acquisition and the processing of the data from the digitizer tablet were carried out by a personal computer (PC-9801, NEC, Tokyo, Japan) using a software package written by one of the authors [29].

RESULTS

Slightly alkaline conditions (pH 7.6–8.0) of the medium which had been kept under an atmosphere containing less than 5% CO_2, caused rounding of the macrophages (Fig. 1). In order to obtain macrophage population where the proportion of the rounded cells, was close to 0.5, CO_2 content of the atmosphere under which incubation was carried out, was kept at a low level of about 2%, throughout the following experiments.

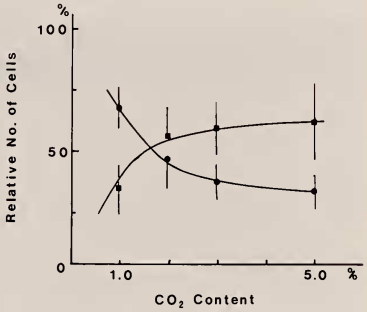

FIG. 1. Percentage of the macrophages in culture with the elongated (■), and the rounded (●) morphology according to the different CO_2 content of the atmosphere. The highly purified phagocytes had been cultured for 24 hr under the designated atmospheric conditions before counting. Each value plotted is the mean of at least 5 independent experiments; vertical bars indicate the standard deviation.

We measured, as stated in Materials and Methods, the rate of LTC_4 production by assaying the rate of the release of the total cysteinyl leukotrienes into the medium using the monoclonal antibodies specific for the peptidolipids. While the antibodies we used, reacted with LTE_4 less strongly than they did so with the other two cysteinyl compounds (see Materials and Methods), the amount of LTE_4 produced by the macrophages under the presently-employed conditions is expected to be negligibly low, owing to the presence of cysteine in the medium: cysteine added to the medium at a concentration of 2 mM, has been shown to inhibit the activity of LTD_4-metabolizing enzyme in the peritoneal macrophages of the rat, by approximately 90% [21]. Therefore, it is perfectly safe and appropriate, as done in the follow-

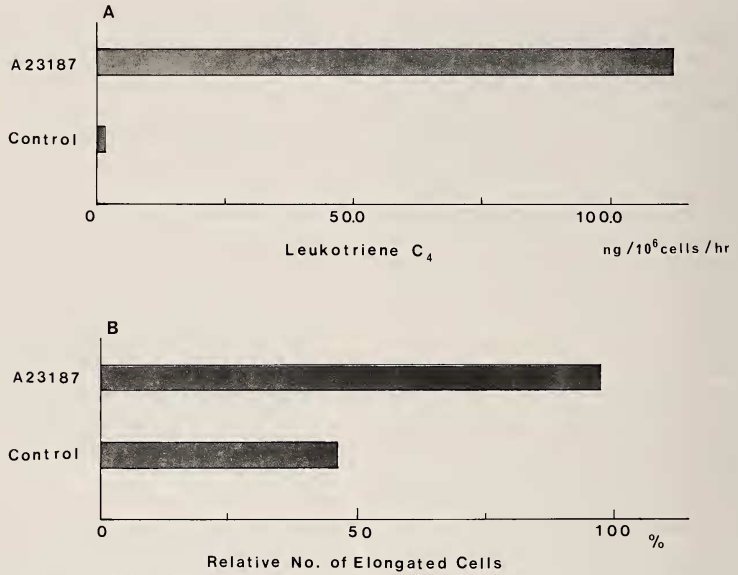

FIG. 2. Effects of calcium ionophore, A23187, upon the rate of LTC_4 production (A) and the cellular morphology (B) in the highly purified macrophages cultured *in vitro*. Concentration of the ionophore was 0.5 μg/ml. The rate of LTC_4 production was calculated, as described in Materials and Methods, on the basis of the values obtained by assaying the total amount of cyteinyl leukotrienes released into the medium during the entire course of 1.5–2.0 hr's incubation period.

ing, to refer to the combined rate of the release of the cysteinyl leukotrienes as representing that of the production of LTC_4 by the cells.

Thus, the highly purified peritoneal macrophages of the mouse in the unstimulated state, under the culture conditions we employed, produced LTC_4 at the rate of 1–3 ng/10^6 cells per hour (Fig. 2A).

However, exposure of the cells to calcium ionophore, A23187, at a concentration of 0.5 μg/ml, for 90–120 min caused approximately hundredfold increase in the rate (Fig. 2A). At the same time, the treatment induced the nearly complete transformation of the macrophages of the rounded cellular shape into the ones with elongated morphology (Figs. 2B, 3).

At 1.0 μg/ml, the ionophore was highly toxic to the cells, and the cells were almost totally disrupted at the end of the 2 hr's incubation period.

At the concentrations below 0.5 μg/ml, the effect of the ionophore rapidly decreased in the dose dependent-manner.

DISCUSSION

The present report demonstrated that calcium ionophore, A23187, brought about the transformation of the cellular morphology in the macrophages of rounded shape and caused them to assume highly elongated form under the alkaline conditions (pH 7.6–8.0) of the medium, while the same treatment induced approximately hundred-

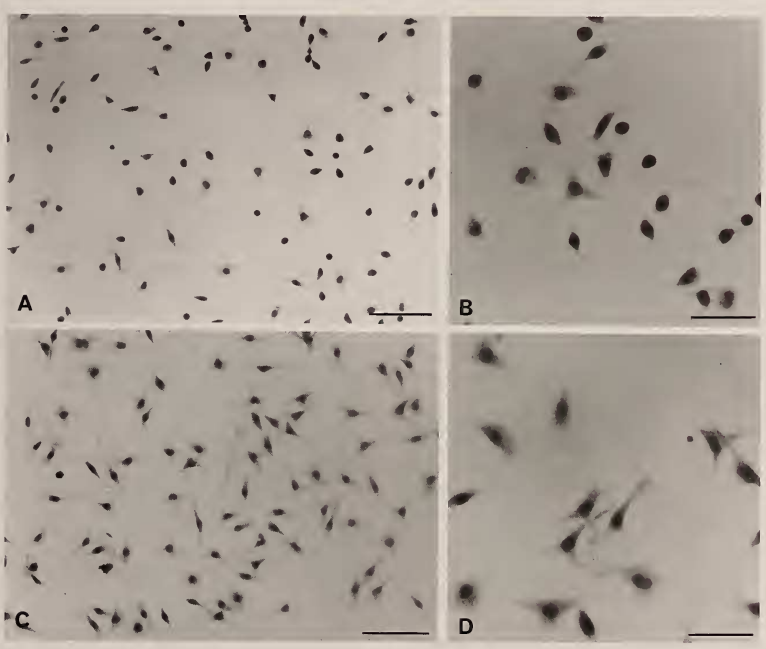

FIG. 3. Photomicrographs showing the appearance of the highly purified macrophages without (A & B) and with (C & D) the ionophore (0.5 μg/ml) treatment. The concentration of the ionophore, and the duration of the incubation period are as described in the legend for Fig. 2. In B and D, the cells are shown at high magnification. Scale indicates 20 μm in A and C, 10 μm in B and D.

fold increase in the rate of LTC_4 production by these cells.

K. Carr and I. Carr [30] analyzed by means of light microscopy, microcinematography and scanning electron microscopy, the morphological properties of the mouse peritoneal macrophages cultured *in vitro*. As a result of their studies, they concluded that the processes passed through by the macrophages in culture, may be divided into 4 consecutive stages, i.e., stages of 1) adhesion, 2) flattening, 3) movement and 4) extension. They suggested, on the basis of microcinematographic studies, that while the moderately elongated cells were moving, the fully extended cells were not actively motile and probably represented a post-motile stage of the cells [30]. Their observations indicated also that the stimulated macrophages attained the fully extended stage earlier in the culture than the non-stimulated ones [30].

The sequence of events followed by the mouse peritoneal macrophages during settlement *in vitro*, under the culture conditions we employed, coincided very well with the description made by K. Carr and I. Carr [30].

Effect of pH upon the spreading (a condition similar to, but probably not identical with the flattening mentioned above) of macrophages in culture was studied by Rabinovitch and DeStepha-

no [31]; they showed that acidic pH of the medium induced spreading of the cells. As described in the present report, the elongation of the macrophages, too, took place under acidic pH while the rounding was caused under alkaline pH of the medium. It might be possible that the spreading and the elongation of macrophages share common cytoskeletal mechanisms underlying the morphological changes.

Relatively little is known about the effects of calcium ionophore upon the cellular shape. Recently, Stanisstreet and Smedley [32] examined the morphological changes of the cells in *Xenopus* embryos following the local application of the ionophre; they found that the treatment caused strong rounding, rather than elongation, of the cells. The cause for the discrepancy between our results and theirs is not known.

Causal relationship between the morphological transformation of the macrophages and the increased rate of LTC_4 production remains to be investigated. Since many of the biological actions of LTC_4 and its metabolites are directed toward or mediated by the smooth muscle cells (for reviews see [4, 5, 9]), it might be possible that LTC_4 and its derivatives produced by the macrophages under the stimulation of A23187, in turn, would affect, either by themselves or in cooperation with PAF (platelet activating factor) [33, 34], the contractile elements in the cells, leading to the changes of their shape. Different mechanisms, however, would have to be postulated for the morphological changes induced by A23187 in the amphibian embryonic cells and those in the mouse macrophages, to account for the discrepancy between our results and those obtained by Stanisstreet and Smedley [32].

Question remains, furthermore, with regard to the relevance of the present findings to the elongation of the macrophages attained spontaneously in culture, or to the physiological processes taking place *in vivo*.

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U. Zor had been on the leave of absence from Dept. Hromone Res., Weizmann Inst. (1986.3 - 1986.5), where he is incumbent of W. B. Graham Professorial Chair of Pharmacology.

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